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Identification of fukinolic acid from Cimicifuga heracleifolia and its derivatives as novel antiviral compounds against enterovirus A71 infection

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Highlights:

- *Cimicifuga heracleifolia* and *Arnebia euchroma* extracts inhibited *in vitro* EV-A71 infection.

- By a combined effort of bioassay-guided purification, high-resolution mass spectrometry and NMR, we identified active peaks from the plant extracts.

- Fukinolic acid and cimicifugic acid A/J and caffeic acid derivatives were deduced as *Cimicifuga heracleifolia* and *Arnebia euchroma*, active compounds, respectively.

- Fukunolic acid derivatives, L- and D-chicoric acid, showed activity in the low micromolar range against EV-A71 clinical isolates.

Title: Identification of fukinolic acid from *Cimicifuga heracleifolia* and its derivatives as novel antiviral compounds against enterovirus A71 infection.

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Abstract

Human enterovirus 71 (EV-A71) infections cause a wide array of diseases ranging from diarrhoea and rashes to hand-foot-and-mouth disease and, in rare cases, severe neurological disorders. No specific antiviral drug therapy is currently available. Extracts from seventy-five Chinese medicinal plants selected for antiviral activity based on the Chinese pharmacopeia and advice from traditional Chinese medicine clinicians were tested for activity against EV-A71. The aqueous extract of the rhizome of *Cimicifuga heracleifolia* (Sheng Ma) and *Arnebia euchroma* (Zi Cao) showed potent antiviral activity. The active fractions were isolated by bioassay-guided purification, and identified by a combination of high-resolution mass spectrometry and NMR. Fukinolic acid and cimicifugic acid A and J, were identified as active anti-EV-A71 compounds for *Cimicifuga heracleifolia*, whereas for *Arnebia euchroma,* two caffeic acid derivatives were tentatively deduced. Commercially available

fukinolic acid analogues like L-chicoric acid and D-chicoric also showed *in vitro* micromolar activity against EV-A71 lab-strain and clinical

isolates.

Introduction

Enterovirus 71 (EV-A71) belongs to the genus *Enterovirus* of the family *Picornaviridae*, small viruses characterized by a naked icosahedral capsid and a positive-sense, single-stranded RNA. EV-A71 is the causative agent of hand-foot-and-mouth disease (HFMD), but other clinical manifestations associated with EV-A71 infection range from skin rashes and herpangina to brain stem encephalitis [1]. Recurrent EV-A71 epidemics of various scales have occurred in the Asia-Pacific region [2, 3]. Because of its marked neurotropism, following the near complete eradication of poliovirus, EV-A71 may become the greatest threat amongst enteroviruses to cause significant neurological complications [4]. An inactived EV-A71 vaccine has been approved in China in 2015 for prevention of HFMD [5]. In view of the rapid recombination of EV-A71, the absence of cross-reactivity among EV-A71 sub-genogroups and the emergence of new genotypes [6, 7], it is not clear whether monovalent vaccines will offer sufficient protection [8]. Moreover, related enteroviruses can cause HFMD, although EV-A71 is the most pathogenic [8]. The main driver for vaccine development has been the lack of an effective antiviral drug. Antiviral drugs are urgently needed to prevent EV-A71 epidemics [9], and drug development has been underway for over a decade [10, 11], targeting various stages of the viral life cycle [12, 13]. There is evidence that some traditional Chinese medicines (TCMs) may be beneficial for the treatment of viral infections, including EV-A71 [14]. Therefore, we decided to test a range of TCM plants for anti-EV-A71 activity. Here, we report the identification of novel anti-EV-A71 compounds from the rhizome of *Cimicifuga heracleifolia* (Sheng Ma) and from *Arnebia euchroma* (Zi Cao).

2. Materials and methods

2.1 Chemicals and compounds

Acetonitrile of HPLC grade, ethanol and acetone of analytical grade were purchased from Sigma-Aldrich Co. (Belgium). Hexane of analytical grade was purchased from Acros Co. (Belgium). Sterile deionized water was produced by a water purification system (Milli-Q Reagent Water System, MA, USA). Rosmarinic acid, L-chicoric acid, D-chicoric acid, salvianolic acid A hydrate, salvianolic acid B, 1,3-dicaffeoylquinic acid, chlorogenic acid and caftaric acid were purchased from Sigma-Aldrich Co. (Belgium).

2.2 Preparation of plant extracts, fractions and compounds

Cimicifuga heracleifolia root and *Arnebia euchroma* were purchased from a certified TCM vendor: Tongrentang Pharm. Co. Ltd. (Beijing, China, http://www.tongrentang.com/en/). The plant material was ground to a fine powder and small-scale extracts were prepared as described before [15]. One gram of plant powder was used in the small-scale extracts. For a large-scale aqueous extract, 100 g of plant powder was transferred into a 1000 mL flask, and sterile deionized water was added in a w/v ratio of 1/10. The flask was then kept at ambient temperature and placed in a bath sonicator for 4 times 15 min each, with an interval of 6 hours. The extract was then centrifuged at 1800 rpm for 8 min. The supernatant was filtered through MN615 185 mm filter paper, transferred into a round-bottom flask and dried in a rotary evaporator (Heto Rotavapor-R). The same volume of fresh deionized water was added to the plant material to start another extraction cycle. A total of three

successive extraction cycles was completed. The dried pooled aqueous extract was then separated on a preparative HPLC column (PrepPak C18, 15 μm, 40x100mm) using acetonitrile/water as mobile phase, whereby the acetonitrile concentration increased linearly to 100%. The elution flow rate was 40 mL/min and controlled by a Waters 600 controller and monitored by a Waters 2487 Dual λ Absorbance Detector at 214 nm and 280 nm. Active compounds were obtained by activity-guided purification. Briefly, active fractions from the preparative HPLC column were separated further using a Sunfire semi-prep C18 column (reversed-phase, 5 μm, 4.6 x 150 mm) with an injection loop of 2 mL on a Shimadzu DGU0A3 system, equipped with a diode array detector monitoring from 190 to 800 nm, and using a gradient elution at a flow rate of 4 mL/min starting with acetonitrile/water at 10%. The HPLC column was washed with 100% acetonitrile for 10 min after each run.

2.3 Mass spectrometry

The analysis was performed on a Q Exactive orbitrap mass spectrometer (Thermo Scientific, San Jose, CA). The mass spectrometer was coupled online to an Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) instrument (Thermo Scientific, San Jose, CA). The UHPLC system was equipped with a 2 μm particle size, 10 nm pore size Easy Spray Pepmap RSLC C18 column with dimensions 50 μm x 15 cm (Thermo Scientific, San Jose, CA). Before sample separation on the analytical column, the lyophilized sample was dissolved in 16 μL of a 2% acetonitrile/0.1% aqueous formic acid (FA) solution. A needle voltage of 1.5 kV and a capillary temperature of 25°C were applied. Next, a 5 μL sample was injected and put on-line with a 3 μm particle size, 10 nm pore size, nanoviper, Acclaim Pepmap 100 C18 precolumn of dimensions 75 μm x 2 cm (Thermo Scientific, San Jose, CA). Sample separation was performed using a 30 min gradient of 0.1% aqueous FA (solvent A)

and 80 % acetonitrile, 0.08 % aqueous FA (solvent B) and a flow-rate of 300 nL/min. The percentage of solvent B increased from 4 % to 70 % in 30 min and was followed by a steep increase to 95 % in 2 min. An inherent rinse step (2 min gradient, from 4 % to 95 % and 3 min rinsing at 95% solvent B) was applied after every run. The Q Exactive mass spectrometer was operated in data-dependent mode. Mass spectra were acquired alternately in the positive and negative ionization mode with an m/z scan range of 100 to 1500 m/z. For each precursor spectrum, up to the ten of the most intense ions were selected for the generation of fragmentation spectra. For precursor spectra, a resolving power of 140,000 full width at half maximum (FWHM) was used with an automatic gain control (AGC) target of 3,000,000 ions and a maximum ion injection time (IT) of 100 msec. For fragmentation spectra, a resolving power of 17,500 FWHM, an AGC target of 1,000,000 ions, an isolation window of 3 m/z, a fixed first mass of 50 m/z and a maximum IT of 256 msec were used. Dynamic exclusion of 10 sec was applied in order to avoid repeated fragmentation of the most abundant ions. Concerning ion selection, only singly-charged ions were selected. From high-accuracy masses, compatible chemical formulae were deduced using ChemCalc [16]. Exact (mono-isotopic) masses or chemical formulae were then used to search databases like Chemspider or Universal Natural Products Database [17] for natural products with these properties. We tentatively identified several compounds by comparing with the published MS/MS profile list in [18-21].

2.4 NMR

The structure of purified compounds was solved by NMR as described before [22]. Briefly, NMR spectra were recorded on 400 MHz and 600 MHz FT-NMR spectrometers in the deuterated solvents and at temperatures indicated in table 2; chemical shifts are expressed in scale (ppm) using tetramethylsilane as an internal standard, and coupling constants J are in Hertz (Hz).

2.5 Antiviral test against EV-A71

Dried extracts or fractions were dissolved in DMSO and tested in a cell-based assay with live enteroviruses as described before [23]. Briefly, RD cells [24], grown for 3-4 days in 150 cm² tissue culture flasks were harvested and seeded in MEM Rega 3 medium (Gibco) supplemented with 2% fetal bovine serum (FBS) (Integro), 1% 200 mM L-glutamine (Gibco) and 1% 7.5% $NaHCO_3$ (Gibco) at a density of 25,000 cells/well in 96-well plates. The microtitre plates were incubated overnight, yielding a non-confluent cell monolayer. Subsequently, the assay plates were transferred to a liquid handling platform on which a sample dilution series was prepared starting at a sample concentration of 100 µg/mL or lower, if necessary. Following preparation of the compound dilution series, virus was added to the wells with a dilution optimized for each EV-A71 strain. The final, maximal DMSO concentration that was reached in the assay wells with the highest sample input (1%) was well tolerated by the cells. The assay plates were returned to the incubator at 37 ℃ for 4 days, at which time the untreated, virus-infected condition (virus controls, VC) showed 100% virus-induced cell death while in the untreated, uninfected condition (cell controls, CC) a perfectly healthy cell

monolayer could be observed. For quantification of the antiviral and anti-metabolic effect of the samples, the assay medium was replaced with 100 µL of a 5% MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, innersalt) (Promega) solution in phenol red-free medium and incubated for 1.5 hours. OD values were recorded with a microtitre plate reader (Safire², Tecan) and uploaded on a custom-designed database-coupled data processing platform (Accelrys) for further processing and quality control.

2.6 Protease assay

A 3C protease assay was performed as previously described [25]. Briefly, COS-1 cells at a concentration of 0.5×10^5 cells/mL, were seeded in a 96-well plate in 100 µl Dulbecco's Modified Eagle's medium (DMEM). The next day, the cells were co-transfected in triplicate using Fugene with 100 ng/well of a construct containing the viral 3C protease-coding region between a Gal4-binding domain and a VP16-activation domain and 100 ng/well of plasmid containing Gal4-sequences upstream of a luciferase reporter under the control of VP16. Two hours after transfection, the cells were treated with DMSO or fukinolic acid-containing medium prepared as a three-fold serial dilution. A rupintrivir-analogue was used as positive control of anti-3C protease activity. After 16 hours post transfection, the medium was removed and the cells were washed with Phosphate-buffered saline (PBS) and lysed in 20 µL Passive Lysis Buffer (Promega) for 30 min at room temperature. The luciferase activity was then measured using the Dual-Glo Luciferase Assay System (Promega) and recorded whenever the 3C-protease activity was affected by the

compound treatment. Therefore, luciferase signal represents, in this assay, a measure of the anti-protease (and in turn antiviral) activity of the selected compounds.

3. Results

3.1 The aqueous extract of *Cimicifuga heracleifolia* and *Arnebia euchroma* inhibits EV-A71 replication *in vitro*

Over seventy-five plants listed in the Chinese Pharmacopeia were selected on the basis of previously reported antiviral activity [26]. Small-scale extracts were prepared from 1 g of botanical material in 10 mL of four different solvents: water, ethanol, acetone and hexane. These extracts were tested in a cell-based antiviral assay to evaluate the *in vitro* antiviral activity against EV-A71. The requirement for the extract to be a true selective inhibitor of EV-A71 replication was the complete protection of the cells from virus-induced cell death for at least one concentration, without causing any significant alteration of cell monolayer morphology as compared to the untreated, uninfected cell control condition. Among the extract tested, *Cimicifuga heracleifolia* aqueous extracts showed strong antiviral activity, together with low levels of cytotoxicity. The EC_{50} was approximately 17 µg/mL and the EC_{90} was 66 µg/mL. Aqueous extracts of *Arnebia euchroma* showed an even more potent activity against EV-A71, with EC_{50} and EC_{90} of 1.6 µg/mL and 5.7 µg/mL, respectively.

3.2 Identification of active fractions by bioassay-guided purification

A large-scale aqueous extract of *Cimicifuga heracleifolia* was prepared as described in materials and methods. The extract was fractionated on a preparative C18 column. Fractions 25-30, 32-33 and 35-40 showed strong anti-EV-A71 activity (Figure 1). In particular, EC_{50}s of fractions 28 and 33 were quite low: around 6.9 and 1.5 µg/mL, respectively. Fractions 28 and 33 were therefore further separated (Figure 1) and tested for anti-EV-A71 activity. Based on the correspondence between active fractions and chromatographic peaks (Figure 1), presumptive active peaks

were collected and their antiviral activity was confirmed. A large-scale aqueous extract of *Arnebia euchroma* was also prepared using the same method. Fractions 34-37, 39-41 and 45-56 showed potent activity against EV-A71 (Figure 2). Particularly, fraction 37 and 39 had an EC_{50} of 7.1 µg/mL and 8.5 µg/mL, respectively. The fractions were further separated using semi-preparative HPLC on a C18 column (Figure 2), and peaks were collected for antiviral testing.

3.3 Fukinolic acid and cimicifugic acid A and J are novel anti-EV-A71 compounds

The antiviral profile of the peaks from fraction 28 of *Cimicifuga heracleifolia* revealed that peak 15 and 16 (F28P15, F28P16) carried the anti-EV-A71 activity with EC_{50} of 28 and 4.3 µg/mL, respectively. Peak 11-14 were isolated from fraction 33 but only the 11 and 13 showed activity (F33P11, F33P13) with EC_{50}s of about 20 µg/mL. After the bioassay-guided purification of the peaks, we wanted to identify the active compounds in each fraction, by means of high-resolution mass spectrometry and NMR. F28P16 was identified as fukinolic acid (Figure 3), first by LC-MS/MS and then further confirmed by NMR (table 1 and S1). F28P15 was tentatively identified to have a chemical formula of $C_{22}H_{22}O_{11}$ and, based on a similar fragmentation pattern than sinapinic acid in MS/MS, it was suggested to be cimicifugic acid J (table 1). Based on MS data, F33P11 had a probable formula of $C_{22}H_{22}O_{12}$ and was identified as cimicifugic acid I by MS/MS and proton NMR. F33P13 was tentatively identified to have a formula of $C_{21}H_{20}O_{11}$ and showed the same fragmentation pattern as cimicifugic acid A/B (table 1). A proton NMR was performed to confirm F33P13 as cimicifugic acid A. Interestingly, the adjacent peak 14 (F33P14), which did not show activity against EV-A71, was identified on the basis of MS/MS and proton NMR as cimicifugic acid B. We were unable to identify F33P11.

Active peaks from *Arnebia euchroma* fraction 37 were peak 3 and 4 (F37P3 and F37P4) with EC_{50} of 12 and 6 µg/mL, respectively: and peak 3 and peak 6 were the active peaks from fraction 39 (F39P3 and F39P6) with EC_{50} of 20 and 9 µg/mL, respectively. Based on MS/MS fragmentation patterns, F37P3 was expected to be a caffeic acid derivative. According to the proposed formula ($C_{27}H_{22}O_{12}$), the active compound could be lithospermic acid, salvianolic acid H/I, or salvianolic acid J (table 1). The presumptive formula of F39P6 ($C_{36}H_{30}O_{16}$) was also deduced from MS. We were unable to get the MS/MS spectrum of F39P6 in negative ion mode, but its positive ion mode MS/MS spectrum was highly similar to F37P3, which suggests that F39P6 may be a conjugate of F37P3, possibly salvianolic acid B, salvianolic acid E or salvianolic acid L. We could not identify F37P6 and F39P.

3.4 Antiviral activity and SAR of fukinolic and caffeic acid derivatives

In order to confirm the bioassay-guided purification and the identification of active compounds, we obtained fukinolic acid and caffeic acid derivatives from commercial sources: L- and D-chicoric acid, rosmarinic acid and salvianolic acid A and B. A cell-based antiviral assay was performed with serial dilution of the purchased compounds in RD cells to confirm/assess their antiviral activity. The results are summarized in table 2. For the fukinolic acid derivatives L- and D-chicoric acid, the activity observed in the active fractions was more pronounced than that observed for the purchased compounds. Salvianolic acid A showed activity in a low µM range, in agreement with a recent publication [27]. By comparing the antiviral activities of the active compounds, we could also deduce some SAR indications. Cimicifugic acid B was the only tentatively identified compound not active *in vitro* against EV-A71. The hydroxyl group at position R2 is thus crucial for antiviral activity (Table

2). In opposition, the hydroxyl group at position R1 is less critical for activity, since the methylation of R1 (cimicifugic acid A) decreased the EC$_{50}$ about 5-fold only. The addition of a hydroxymethyl group at position R3 (cimicifugic acid I) also resulted in loss of activity. Ultimately, although cimicifugic acid J shares the same R groups as cimicifugic acid I, it showed some activity against EV-A71 suggesting that the substituents in the opposite benzyl play also a role in the activity of this class of compounds.

3.5 Fukinolic and caffeic acid analogues showed potent antiviral activity against EV-A71 clinical isolates

In order to explore the clinical relevance of our findings, we wanted to assess the activity of the aforementioned fukinolic and caffeic derivatives in the context of EV-A71 clinical isolates infection. Indeed, the lab-adapted strain BrCr used for the previous experiment is poorly representative of the circulating EV-A71. For instance, clinically relevant genogroup B and C were used in this experiment to infect RD cells in the presence of serial dilution of compounds. Pirodavir, a capsid binder, was used as a reference compound. Overall, all the tested derivatives showed a potent activity, in a low micromolar range, against EV-A71 clinical isolates with an improvement up to 50x compared to the activity against the lab-adapted BrCr strain (table 3). The antiviral activity of fukinolic and caffeic acid derivatives was also assessed in the context of non-EV-A71 enteroviruses (rhinovirus B14 and coxsackievirus B3) and non-enteroviruses infection (Zika virus and Chikungunya virus). The compounds proved inactive against all tested viruses, suggesting that these compounds are selective inhibitors of EV-A71 replication.

3.6 Inhibition of 3C protease activity

Fukinolic acid and cimicifugic acid J have been previously described to inhibit (carboxyl) peptidases [28]. We therefore wanted to assess the effect of fukinolic acid on the activity of the 3C viral peptidase. We hypothesized that the anti-protease activity could explain the antiviral effect of fukinolic acid and its analogues on EV-A71 replication. A 3C protease activity assay was performed by co-transfecting COS-1 cells with a construct encoding the 3C protease gene flanked by a Gal4 binding domain and a VP16 domain and a luciferase construct with an upstream Gal4 sequence and under the control of the VP16 domain [25]. Briefly, if the 3C protease is functional it will cleave, with its auto-catalytic domain, the Gal4BD and the VP16 domain and the luciferase reporter will become transcriptionally inactive (no luciferase signal). On the other hand, if the activity of the 3C protease is affected (by the compound treatment) the Gal4BD-3C-VP16 ORF will bind the Gal4 sequence on the luciferase reporter plasmid and the VP16 domain will recruit the RNA polymerase to start the transcription of the reporter (luciferase signal). Rupintrivir is a well described viral 3C protein inhibitor and a rupintrivir-analogue was used as positive control. Following co-transfection and treatment with serial dilution of the rupintrivir-analogue and the fukinolic acid, we detected an increased luciferase signal, i.e an increased anti-protease activity only following rupintrivir-analogue treatment (Figure 3). Fukinolic acid did not show any anti-protease activity suggesting that it inhibits viral replication with a different mechanism of action.

4. Discussion

Cimicifuga heracleifolia has been used in the clinic to treat flu-like illness and sore throat for centuries [29]. *Cimicifuga heracleifolia* is also a component of Sheng-Ma-Ge-Gen-Tang (SMGGT), a popular complex prescription used to treat measles in children. SMGGT proved active also against EV-A71 *in vitro* [30]. Fukinolic acid and several cimicifugic acids were isolated from some *Cimicifuga* species before [31]. We reported here for the first time that fukinolic acid, as well as cimicifugic acid A and J are the anti-EV-A71 active compounds from *Cimicifuga heracleifolia.* We also evaluated the antiviral activity of fukinolic acid analogues against EV-A71 clinical isolates and we detected a remarkable improvement of antiviral activity as compared to the lab-adapted BrCr strain, emphasizing the clinical relevance of our findings. Fukinolic acid analogues like L-chicoric and D-chicoric acid, and dicaffeoylquinic acid were found to have anti-human immunodeficiency virus (HIV) integrase activity [32]. Since EV-A71 replication is purely cytoplasmic and does not rely on an integrase activity, the antiviral activity of the here-described class of compounds must be mechanistically distinct. We tested instead the anti-EV-A71-3C-protease activity of fukinolic acid in a cell-based 3C protease assay but the result of the assay was negative suggesting that this mechanism of action is unlike.

Arnebia euchroma is a species of the *Boraginaceae* family, whose roots are often used to regulate blood circulation in TCM [33]. The extracts from plants of the *Boraginaceae* family have various biological activities like antifungal [34], antioxidant [33] and anti-HIV activities [35]. Caffeic acid analogues like rosmarinic acid and lithospermic acid were identified from plants of the *Boraginaceae* family before [36, 37], and also from *Salvia miltiorrhiza* [21], whose aqueous extract also shows activity against EV-A71 [38]. Rosmarinic acid, lithospermic acid and

lithospermic acid B have shown *in vitro* anti-HIV activity [39]. Moreover, it has been reported that rosmarinic acid, salvianolic acid A, salvianolic acid B and magnesium lithospermate B have anti-EV-A71 activity [27]. In this study, we confirmed these finding and we explore the activity of salvianolic acid in the context of infection by clinically-relevant EV-A71 strains. As previously mentioned, rosmarinic, lithospermic and various salvianolic acids (included A and B) were identified from Salvia miltiorrhiza (Danshen) before [21, 40] whose aqueous extract also shows activity against EV-A71 [38]. Danshen has been widely used in China for centuries. Many recent clinical trials of Danshen products support its safe use in humans [40] and Danshen Capsule has received marketing authorisation in The Netherlands as a herbal remedy[1]. Combined with our *in vitro* findings, these facts support the potential use of Danshen for the treatment of EV-A71-infected patients.

Conclusion

With a combined effort of bioassay-guided purification, high-resolution mass spectrometry and NMR, we identified the fractions of *Cimicifuga heracleifolia* and *Arnebia euchroma* responsible for anti-EV-A71 activity *in vitro*. For the first time, we described fukinolic acid, as well as cimicifugic acid A and J as inhibitors of EV-A71 replication. Moreover, we report a very pronounced activity of fukinolic acid derivatives (in the low micromolar range) in the context of EV-A71 clinical isolates infection. We also identified two caffeic acid derivatives as active compounds against EV-A71. Efforts to determine the precise mechanism of action of these classes of compounds are currently ongoing.

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[1] https://english.cbg-meb.nl/

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Declarations

Competing Interests: None

Ethical Approval: Not required

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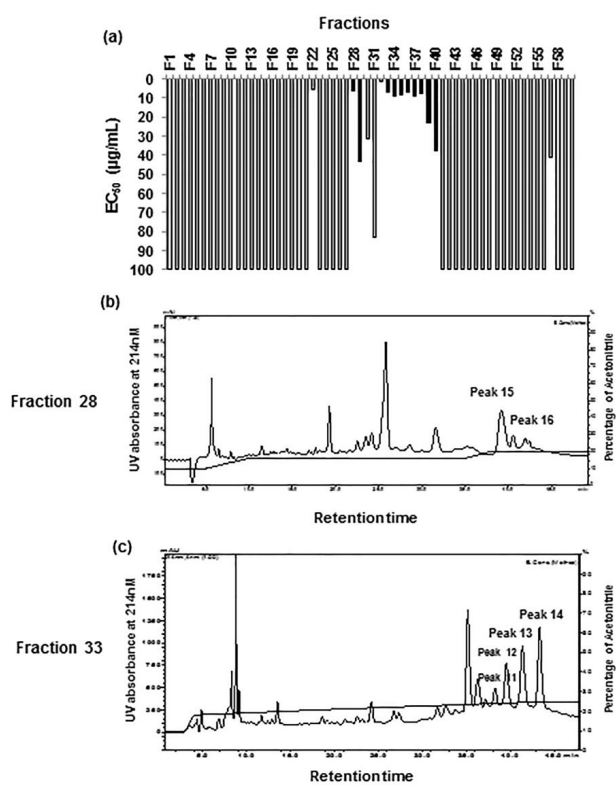
FIGURE LEGEND

Figure 1. (a) The anti-EV-A71 activity-guided separation and purification of *Cimicifuga heracleifolia* fractions. Sixty fractions (F1-F60) were obtained and tested for activity against the EV-A71 BrCr strain. When at least at one concentration a fraction could fully inhibit the virus-induced CPE without showing toxicity, the corresponding bar is marked in black (non-active fractions are depicted in gray). (b,c) HPLC chromatogram of fraction 28 and fraction 33. F28 and F33 were chosen for further purification on a semi-preparative C18 HPLC column using a gradient elution as indicated by the black line. Peaks were collected and tested for anti-EV-A71 activity. F28 peak 15, F28 peak 16, F33 peak 11 and F33 peak 13 were active. EC_{50}: concentration of compound at which the virus-induced cytopathic effect is reduced by 50%. Values are from one experiment.

(a)

Fractions

(b) Fraction 28

(c) Fraction 33

Figure 2. (a) The anti-EV-A71 activity-guided separation and purification of *Arnebia euchroma* fractions. Sixty fractions (F1-F60) were obtained and tested for activity against the EV-A71 BrCr strain. When at least at one concentration a fraction could fully inhibit the virus-induced CPE without showing toxicity, the fraction bar is marked in black (non-active fractions are depicted in red). (b,c) HPLC chromatogram of fraction 37 and fraction 40. F37 and F40 were chosen for further purification on a semi-preparative C18 HPLC column using a gradient elution as indicated by the black line. Peaks were collected and tested for anti-EV-A71 activity. F37 peak 3, F37 peak 4, F40 peak 3 and F40 peak 6 were active. EC_{50}: concentration of compound at which the virus-induced cytopathic effect is reduced by 50%. Values are from one experiment.
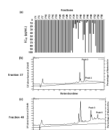
Figure 3. Anti-EV-A71 3C protease activity of fukinolic acid. Cell-based EV-A71 3C protease assay was performed by co-transfecting a plasmid encoding for EV-A71 3C protease in combination with a pG5luc reporter plasmid. Fukinolic acid, rupintrivir-like (positive control) and DMSO (negative control) were added and after 24h, the luciferase levels were measured. The inhibition of protease activity was measured as an induction of FLuc expression and subsequent increase of luciferase signal. Experiments were performed in triplicate and values represent the mean ± SD of the three measurement.
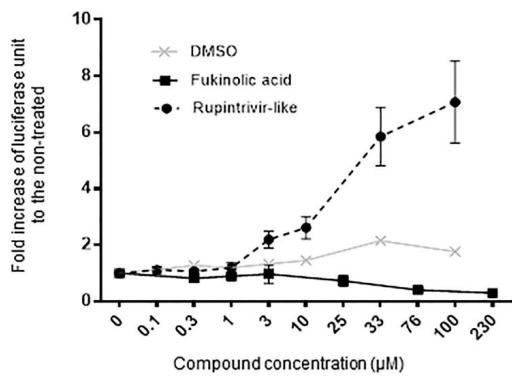
Table 1. Characterization by mass spectrometry and anti-EV71 activity of active peaks.

Plant	Peak	[M-H]⁻ m/z	[2M-H]⁻ m/z	[M+H]⁺ m/z	[2M+H]⁺ m/z	HPLC-ESI-MS² m/z (% base peak)	Tentatively identified compounds	EC$_{50}$ ±SD (μg /mL)	CC$_{50}$±SD (μg /mL)
Cimicifuga heracleifolia	F28P15	-	-	463.1233	925.2380	MS²[463]: 224(1), 207(100), 175(27), 147(3), 119(2), 91(1)	Cimicifugic acid J[a]	27.7±1.1	>70
	F28P16	433.0763	867.1613	435.0922	869.1769	MS²[433]: 433(1), 271(98), 253(35), 235(7), 209(3), 191(27), 179(100)	Fukinolic acid[a,c]	4.3±0.1	50±0
	F33P11	-	-	-	-	-	-	26.9±15.9	>100
	F33P12	477.1041	-	479.1159	957.2229	MS²[477]: 253(70), 223(10) 191(100), 181(75), 165(50), 109(30)	Cimicifugic acid I[c]	>100	>100
	F33P13	447.0920	-	449.1070	914.2378	MS²[447]: 447 (100), 253 (90), 235(5), 191 (10), 181 (10)	Cimicifugic acid A[a,c]	21.3±11	>100
	F33P14	447.0929	-	449.1068	914.2314	-	Cimicifugic acid B[c]	>100	>100
Arnebia euchroma	F37P3	537.0637	1075.1331	-	-	MS²[537]: 339(20), 295(23), 269(22),228(23), 197(100), 179(55), 135(95), 109(8)	C$_{27}$H$_{22}$O$_{12}$[b]	10.6±0.4	>90
	F37P4	-	-	-	-	-	.	5.4±1.1	>100
	F39P3	-	-	-	-	-	-	19.8±2.3	>100
	F39P6	717.0912	1435.1892	-	-	-	C$_{36}$H$_{30}$O$_{16}$[b]	36.3±3.0	>100

[a] Identification by comparing with previous published report.
[b] Identified as caffeic acid derivatives based on MS/MS fragmentation patterns.
[c] Identification confirmed by NMR spectrum.

EC$_{50}$, concentration of compound at which the virus-induced cytopathic effect is reduced by 50%.

CC$_{50}$, concentration of compound at which a 50% reduction in cell viability is observed.

EC$_{50}$ and CC$_{50}$ values were obtained from multiple independent (>1) experiments.

Table 2. Summary of compound structures and their activities against EV71 BrCr lab strain.
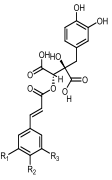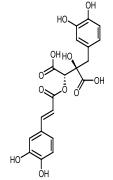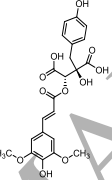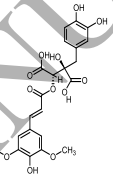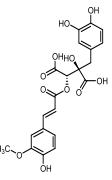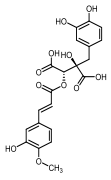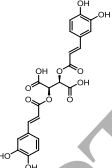
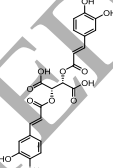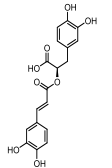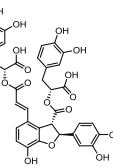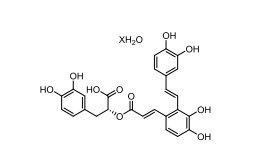|	Name	Core structure	Fukinolic acid	Cimicifugic acid J	Cimicifugic acid I	Cimicifugic acid A	Cimicifugic acid B
Structures of tentatively identified compounds from *Cimicifuga heracleifolia*	$EC_{50} \pm SD$ (μg /mL)	/	4.3±0.1	27.7±1.1	>100	21.3±11	>100
	Structure						
	Name	L-Chicoric acid	D-Chicoric acid	Rosmarinic acid	Salvianolic acid B	Salvianolic acid A hydrate	
Structures of compounds obtained commercially	$EC_{50} \pm SD$ (μg /mL)	55.5±10.8	60.7±7.4	21.9±2.1	55.5±11.1	3.6±0.2	
	Structure						

Table 3. Antiviral activity of commercially obtained compounds against EV71 BrCr and clinical isolates.

EV71 Genogroup	Virus strain	$EC_{50} \pm SD$ (µg /mL)					
		L-Chicoric acid	D-Chicoric acid	Rosmarinic acid	Salvianolic acid A	Salvianolic acid B	Pirodavir
A	BrCr	55.5±10.8	60.7±7.4	21.9±2.1	3.6±0.2	55.5±11.1	1.2±0.1
B2	11316	9.0±0.4	9.1±0.9	3.2±0	0.7±0.1	3.0±0.5	0.2±0.02
B5	TW/70902/08	10.6±2.6	8.3±0.4	3.8±0.1	1.1±0	4.3±0.3	0.2±0.02
C2	H08300 461	7.6±0.8	8.4±0.1	3.9±0.1	0.9±0	1.1±0.2	0.3±0.03
C4	TW/1956/05	8.4±0.2	8.4±0.1	2.2±0.1	1.1±0	2.1±0	0.2±0
CC_{50} (µg /mL)		>100	>100	56.4±7.1	25.5±7.4	86.4±7.6	>10